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19 ABSTRACT *(Continue on reverse if necessary and identify by block number)*

We have developed systems for genetic analysis of halophilic archaebacteria. These include (i) transformation systems which we have shown to work efficiently in at least three genera of halobacteria, (ii) shuttle vectors which can be selected in either halobacteria or *E. coli*, and (iii) host systems including hundreds of mutant halobacterial strains. We have applied these methods of genetic analysis in characterizing a halobacterial HMGCoA reductase gene (which is also the basis of our selection for antibiotic resistance in halobacteria) and clusters of genes involved in the biosynthesis of tryptophan, histidine, arginine and leucine. These techniques have also permitted us to position dozens of genetic markers on a physical map of the *Haloferax volcanii* genome. Other applications include the construction of halobacterial suppressor tRNA genes by site directed mutagenesis and cloning of genes encoding halobacterial membrane proteins.

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PRINCIPAL INVESTIGATOR: W. Ford Doolittle

CONTRACTOR: Dalhousie University
                               Halifax, Nova Scotia

CONTRACT TITLE: Development of Shuttle Vectors for Halobacteria

PERIOD OF PERFORMANCE: 1 February 1988 - 31 January 1991

OBJECTIVE: Develop transformation systems for halophilic archaebacteria, construct a variety of useful halobacterial plasmid vectors including halobacteria - *E. coli* shuttle vectors, and construct suitable halobacterial host strains. Demonstrate the utility of these genetic tools with several model applications.

ACCOMPLISHMENTS:

Transformation. During the course of this contract we have developed efficient and broadly applicable PEG-mediated spheroplast transformation systems for halophilic archaebacteria including *Halobacterium halobium*, *Haloferax volcanii* and *Haloarcula hispanica*. Using these methods we have demonstrated transfection with phage DNA and transformation with linear genomic DNA or plasmid DNA (including shotgun-cloning ligations). Transforming DNA can be either single or double stranded. Spheroplast regeneration under our conditions is also very efficient.

At this point we have performed thousands of transformations and the techniques can be considered completely routine. We have extended our basic protocols to include a microtiter plate-based mini transformation protocol for conveniently performing very large numbers of transformations and a quick transformation protocol which uses cells simply scraped from the surface of plates. In addition, we have developed convenient procedures for storing frozen cells for transformation.

Basically the transformation method can be summarized in six steps: (i) Resuspend cells in spheroplasting solution, (ii) Form spheroplasts by adding EDTA, (iii) Add DNA, (iv) Add PEG solution, (v) Dilute or wash cells with regeneration salts solution, (vi) Plate cells in regeneration top agar.

While our transformation methods work well for representatives of at least three halobacterial genera, we have found that some halobacterial species (*Halobacterium saccharovorum*, *Halobacterium trapanicum*, *Halococcus morrhuae*) are refractory to spheroplast formation by treatment with EDTA and therefore appear to be unsuitable for transformation by this method.

The bulk of our work has been performed using *Hf. volcanii* as a model organism. We have made extensive use of non-replicating homologous DNAs (genomic DNA, cosmid clones, restriction fragments from agarose gels, etc.) in transforming *Hf. volcanii*. Transformations with these DNAs require recombination with the genome and we have demonstrated that this is a reasonably efficient process in *Hf. volcanii*. Typically such experiments involve transformation with wild-type DNA and selection for correction of an auxotrophy, or the use of directly selectable markers such as drug resistance.

Generally, DNAs propagated in foreign hosts are restricted by a factor of about 10^4-fold by *Hf. volcanii*, but, thanks to the high efficiency of the transformation system, this has not hindered our work. We have recently learned that plasmid DNA which has been passaged through a *dam*⁻ *E. coli* strain (such as JM110) evades restriction by *Hf. volcanii* (M.L. Dyall-Smith and S.N.

Nuttall, University of Melbourne, personal communication). We have confirmed that the efficiency of transformation of *Hf. volcanii* by JM110-propagated plasmid DNA can exceed 10^8 per microgram, compared to 10^4 - 10^5 transformants per microgram with plasmid DNA propagated in dam^+ strains such as DH5α.

Vector development. We have developed a series of *E. coli*-halobacterial shuttle vectors. The work horse among these has been the 10.5 kb pWL102 (see figure on highlight page). This vector carries (i) a ColE1 replicon and ampicillin resistance from pAT153, (ii) part of the sequenced *Hf. volcanii* plasmid pHV2 (iii) a number of useful cloning sites and (iv) a 3.5 kb fragment conferring resistance to mevinolin (a competitive inhibiter of HMGCoA reductase) in *Hf. volcanii*.

Early attempts at constructing shuttle vectors using a pUC-type vector as an *E. coli* replicon resulted in some instability in *E. coli*. Instability problems were completely overcome by relying on pAT153, with its lower copy number, as the basis for replication in *E. coli*.

Halobacterial replication functions of pWL102 are carried by 3747 bp representing about 60% of the parental pHV2 sequence. This DNA contains an ORF capable of encoding a polypeptide of 808 amino acid residues flanked by 1323 bp which include 532 bp of the 5-prime portion of a 600 bp ORF predicted in the original pHV2 sequence. Presumably this amount of DNA includes sufficient information for replication control and maintenance as well as the origin of replication.

Evidence suggests that pWL102 is not transmissible. Rosenshine et al. (Science 245:1387-1389, 1989) have relied on pHV2 as an immobile cytoplasmic marker in *Hf. volcanii* mating experiments. The shuttle vector cannot be mobilized in *E. coli* either since the ColE1 origin for conjugal transfer was deleted during the original construction of pAT153.

The mevinolin resistance (Mev^R) locus of pWL102 was originally acquired from a spontaneous mutant of *Hf. volcanii*. We have sequenced the Mev^R locus, as well as its wild-type counterpart, and found that it has a high degree of sequence similarity to eukaryotic genes for HMGCoA reductase but is very unlike a eubacterial example of this gene from *Pseudomonas*. The 1212 bp gene is about half the size of coding portions of typical eukaryotic homologs since its product, being a soluble enzyme in *Hf. volcanii*, lacks the eukaryotic membrane spanning domains. The point mutation which confers resistance to mevinolin has been identified as a change from GTTA to TTTA 29 bases upstream of the initiating ATG in a region with similarity to a predicted consensus sequence for archaebacterial promotors (see figure on highlight page). This appears to be a first *in vivo* example of an archaebacterial promotor mutation (other than insertional inactivation) and, as such, should yield further insight into the structure of archaebacterial promotors. Increase in HMGCoA reductase levels by a promotor "up" mutation is consistent with our finding that *Hf. volcanii* can also overcome the inhibition of this enzyme by mevinolin through a second mechanism: amplification of the HMGCoA reductase gene.

Sequence information is available for about 85% of pWL102 including all of the pHV2 and pAT153 portions and about 2 kb of the Mev^R insert. Unique restriction sites are present for Kpn I, Xba I, Eco RI, Cla I, Bam HI, Nco I and Sph I. Insertions can be made into any of these sites without disrupting plasmid maintenance or resistance functions.

Our shuttle vectors have been distributed to a number of other laboratories. Importantly, our *Hf. volcanii* mevinolin resistance locus has provided selection for a *Hb. halobium-E. coli* shuttle vector, pUBP2, constructed by F. Pfeifer and U. Blaseio (Proc. Natl. Acad. Sci. USA 87:6772-6776, 1990). Pfeifer's group has found that pUBP2 replicates in *Hf. volcanii* as well as *Hb. halobium* and we have employed this vector ourselves in experiments with the *Hb. halobium* bacteriorhodopsin gene (see below). Our early experiments indicated that *Hb. halobium* could not be stably transformed with pWL102. Pfeifer's group also found this to be the case, but, surprisingly, when they inserted a *Hb. halobium vac* gene into the pWL102 Kpn I site (between the Mev^R insert and the pHV2 sequence) stable replication in *Hb. halobium* resulted. We subsequently demonstrated that both pWL102 and pUBP2 replicate stably in *Ha. hispanica*, a halophile somewhat more distantly related to *Hb. halobium* and *Hf. volcanii* than either of these two species are to one another. Thus the halobacterial replicons of both of these shuttle

vectors appear to have broad host ranges, transforming representatives of at least three halobacterial genera. This is roughly comparable to the phylogenetic depth spanned by broad host range vectors such as RSF1010 or RP4 within the γ subdivision of the purple bacteria.

Other shuttle vectors which we have constructed include those bearing (instead of the mevinolin-resistance determinant) (i) the *Hf. volcanii* homolog of the *E. coli trpB* gene which is selectable in *Hf. volcanii trpB* mutants or (ii) *Hf. volcanii* tRNA^{Ser}-derived amber, ochre or opal suppressors which are potentially selectable in *Hf. volcanii* nonsense mutants.

Host development. Initially, we cured wild-type *Hf. volcanii* of its endogenous pHV2 to create an appropriate host for transformation with pHV2-derived vectors. This strain, WFD11, has served as the parent for virtually all of our subsequent strain constructions. EMS mutagenesis of WFD11 was followed by screening and identification of 256 auxotrophs in the biosynthetic pathways for 16 amino acids as well as pathways for biotin, adenine, guanine, and pyrimidine biosynthesis. Double mutants have also been generated. We have an additional 126 auxotrophs which are defective in unidentified pathways. All of these and others, such as our Mev^R mutants, spontaneous amino acid analog resistance mutants, and a number of *Hf. volcanii* strains from the collection of M. Mevarech (Tel Aviv University), are being maintained as frozen stocks in a well organized collection with a companion computerized data base.

Our vector and transformation systems have been integrated into ongoing work in this laboratory to create a physical map of the *Hf. volcanii* genome using bottom-up mapping by ordering a set of cosmid clones and top-down mapping of restriction fragments by pulsed field gel electrophoresis (mapping work supported by Medical Research Council). By transforming the EMS-generated auxotrophic mutants mentioned above with a minimal ordered set of about 160 *E. coli* cosmid clones of the *Hf. volcanii* genome, we have succeeded in genetic mapping of 139 alleles to 28 different clusters. These include alleles representing arginine, glutamine, histidine, isoleucine-valine, leucine, lysine, methionine, serine, threonine, tryptophan, tyrosine, early aromatic, adenine, guanine and pyrimidine biosynthetic pathways. We have also mapped the HMGCoA reductase gene genetically as well as several of those auxotrophic alleles defective in unidentified pathways.

We have characterized (by subcloning and sequencing) cosmid clones which complement auxotrophies in the tryptophan, arginine, histidine and leucine pathways. This has allowed us to identify homologs of the *E. coli* genes *hisC, trpD, trpF, trpE, trpG, trpC, trpB, trpA, argF, argB, argC* and *pyrB*, as well as *oat* (encoding ornithine acetyltransferase), a gene not found in *E. coli*.

The *trpCBA* and *trpDFEG* clusters were found to map to separate locations in the genome. The gene order for both clusters was unique compared with eubacterial examples and distinct from an example of methanogen organization as well. Genes in both *trp* clusters contain overlapping start and stop codons. The *trpCBA* cluster is preceded by sequence which can potentially be folded to form alternate stable stem-and-loop structures, and we're now taking a look at whether these may play some regulatory role

We mapped the arginine mutations of 20 auxotrophs to one cosmid. Sequencing of DNA which complemented arginine auxotrophies revealed the following organization: -ORF-> -*argC*-> -*argF*-> -*argB*-> -*oat*-> -URF-> -*pyrB*->. Again, several of these genes have overlapping start and stop codons and the remaider have abutted stop/start codons. The presence of *oat* is diagnostic of a more energy efficient pathway compared to that found in some eubacteria such as *E. coli* and *B. subtilis*, which lack it. This enzyme has also been found to be present in methanogens and thermophiles.

The genetic defects in 18 of our histidine auxotrophs have been mapped to four chromosomal locations (i.e. mapped to non-overlapping cosmid clones). Sequencing of 2 kb of DNA from one of these complementing clones revealed a 1086 bp (361 aa) homolog to the *E. coli hisC* gene (encoding histidinol-phosphate aminotransferase). There do not appear to be additional genes near the *hisC* gene as judged by examination of ustream and downstream sequence.

About two dozen leucine auxotrophs have been characterized by growth on pathway intermediates into at least three classes. All of these map to one cosmid (~40 kb of *H. volcanii*

DNA). Three kilobases of DNA from this cosmid has been cloned into the shuttle vector pWL102 and shown to complement several auxotrophs. This DNA is being sequenced.

These detailed characterizations have lead to the ability to perform sophisticated host constructions in *Hf. volcanii* such as deletion of the genome's *trpB* region thus creating a host lacking sequence homology with the *trpB* shuttle vector referred to above.

Special Applications. *Suppressor tRNAs.* Using site-directed mutagenesis we have changed the anticodon of a cloned *H. volcanii* tRNA^{Ser} gene to be complementary to the three nonsense codons, thus creating potential suppressor tRNAs. A 248 bp sequence including the tRNA gene has been cloned into pWL102 for each of the suppressor tRNAs plus the wild type. Thus each plasmid is isogenic except for the bases encoding the anticodon. Preliminary indications are that the UAG suppressor plasmid is lethal in *Hf. volcanii.* We are currently screening our collection of auxotrophs for nonsense mutants by transformation with the UAA and UGA suppressor tRNAs. Once we've identified nonsense mutants we will try using these as hosts for shuttle vectors which only rely on the cloned suppressor tRNAs for selection (as mentioned above).

Membrane proteins. In work being done in collaboration with J. Spudich (Albert Einstein College of Medicine, New York), and based on his observation that *Hf. volcanii* is motile, we have located probable *Hf. volcanii* sensory rhodopsin and flagellin genes by probing restriction digests of cosmid clones of the *Hf. volcanii* genome with the *Hb. halobium sopI* and *flaB* genes (*sopI* clone provided by D. Oesterhelt, Max-Planck-Institut für Biochemie, Martinsried; *flaB* clone provided by M. Sumper, Universität Regensburg). We are in the process of cloning these with the ultimate goal of functional analysis by construction of site specifically altered genes.

We have used pWL102 and the *Hb. halobium* shuttle vector pUBP2 to clone a 9 kb fragment carrying the *Hb. halobium* bacteriorhodopsin gene cluster (supplied by M. Betlach, University of California, San Francisco). These constructs transformed the Bop⁻ *Hb. halobium* strain Pho81 to a Bop⁺ phenotype. *Hf. volcanii* and *Ha. hispanica*, species which do not naturally produce purple membrane, were also transformed with these constructs, but, surprisingly, transformants did not appear to express bacteriorhodopsin even when grown in media containing retinal. Since the DNA cloned in these shuttle vectors includes all of the genes presently known to be involved in bacteriorhodopsin expression, our results suggest that there may remain further genetic elements to be recognized. *Hf. volcanii* and/or *Ha. hispanica* may constitute useful genetic backgrounds for determining what additional genetic elements are required for expression.

PUBLICATIONS:

We list only publications related to this contract. Those marked with asterisks were submitted since ONR support began, and acknowledge this support.

84.   Cline, S.W. and Doolittle, W.F. (1987)
        Efficient transfection of the archaebacterium *Halobacterium halobium.* J. Bacteriol.
        169:1341-1344.

89.   Charlebois, R.L. Lam, W.L., Cline, S.W. and Doolittle, W.F. (1987)
        Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating
        transformation of an archaebacterium. Proc. Natl. Acad. Sci. USA 84:8530-8534.

94.   Charlebois, R.L. and Doolittle, W.F. (1989)
        Transposable elements and genome structure in halobacteria. in Mobile DNA M. Howe
        and D. Berg, eds. American Society for Microbiology, pp. 297-307.

95.*   Cline, S.W., Lam, W.L., Charlebois, R.L., Schalkwyk, L.C. and Doolittle, W.F. (1989)
        Transformation methods for halophilic archaebacteria. Can. J. Microbiol. 35:148-152.

96.* Charlebois, R.L., Hofman, J.D., Schalkwyk, L.C., Lam, W.L. and Doolittle, W.F. (1989) Genome mapping in halobacteria. Can J. Microbiol. 35:21-29.

98.* Lam, W.L. and Doolittle, W.F. (1989)
Shuttle vectors for the archaebacterium *Halobacterium volcanii*. Proc. Natl. Acad. Sci. USA 86:5478-5482.

99.* Cline, S.W., Schalkwyk, L.C., and Doolittle, W.F. (1989)
Transformation of the archaebacterium *Halobacterium volcanii* with genomic DNA. J. Bacteriol. 171:4987-4991.

100.* Lam, W.L., Charlebois, R.L. and Doolittle, W.F. (1990)
Progress in the molecular biology of the archaebacteria. UCLA Symp. Mol. Cell. Biol. (New Series) 122:265-272.

101.* Doolittle, W.F., Charlebois, R.L., Schalkwyk, L.C., Lam, W.L., Conover, R.K., Tsouluhas, D., Cline, S.W., Hofman, J.D. and Cohen, A. (1991)
Physical mapping and gene transfer methods for *Halobacterium (Haloferax) volcanii*. in General and Applied Aspects of Halophilic Microorganisms, F. Rodriguez-Valera, ed. Plenum, pp. 271-276.

102.* Conover, R.K. and Doolittle, W.F. (1990)
Characterization of a gene involved in histidine biosynthesis in *Halobacterium (Haloferax) volcanii*: isolation and rapid mapping by transformation of an auxotroph with cosmid DNA. J. Bact. 172:3244-3249.

103.* Lam, W.L., Cohen, A., Tsouluhas, D. and Doolittle, W.F. (1990)
Genes for tryptophan biosynthesis in the archaebacterium *Haloferax (Halobacterium) volcanii*. Proc. Natl. Acad. Sci. U.S.A. 87:6614-6618.

PhD THESES: Two PhD theses relate to work conducted under this contract.

Charlebois, R.L. 1987. Characterization of pHV2 from *Halobacterium volcanii* DS2 and its use in demonstrating transformation of an archaebacterium. PhD Thesis, Dalhousie University.

Lam, W.L. 1991. Genetic Analysis in *Haloferax volcanii*. PhD Thesis, Dalhousie University.

MANUSCRIPTS IN PREPARATION:

Cline, S.W. and Doolittle, W.F. (1991) Transformation of members of the genus *Haloarcula* with shuttle vectors based on *Halobacterium halobium* and *Haloferax volcanii* plasmid replicons. In preparation for J. Bacteriol.

Cohen, A., Lam, W.L., Cline, S. W. and Doolittle,W.F. (1991) Genetic map of the halophilic archaebacterium *Haloferax volcanii*. In preparation for Proc. Natl. Acad. Sci. USA.

Conover, R.K. and Doolittle, W.F. (1991) Characterization of an arginine operon in *Haloferax volcanii*. In preparation for J. Bacteriol.

Lam, W.L. and Doolittle, W.F. (1991) Structural and functional conservation between archaebacterial and eukaryotic 3-hydroxy-3-methylglutaryl coenzyme A reductases. In preparation for Nature.

Lam, W.L., Logan, S.M. and Doolittle, W.F. (1991) Structure and organization of the genes for tryptophan biosynthesis in the archaebacterium *Haloferax volcanii*. In preparation for J. Bacteriol.

Lam, W.L., Charlebois, R.L. and Doolittle, W.F. (1991) Mechanisms for mevinolin resistance in the archaebacterium *Haloferax volcanii*: overexpression of 3-hydroxy-3-methylglutaryl coenzyme A reductase. In preparation for J. Biol. Chem.

Lam, W.L., Logan, S.M. and Doolittle, W.F. (1991) Gene replacement and gene deletion in *Haloferax volcanii*. In preparation for J. Bacteriol.

REPORTS: Aspects of work performed under this contract have been presented at the following meetings.

Third International Symposium on the Molecular Biology of the Archaebacteria, Victoria, British Columbia, 1988.

Sixteenth International Congress of Genetics, Toronto, Ontario, 1988.

UCLA Symposium on Molecular Evolution, Lake Tahoe, California, 1989.

Canadian Institute for Advanced Research Genome Evolution Meeting, Victoria, British Columbia, 1989.

Molecular Evolution Course, Wood's Hole, Massachusetts, 1989.

FEMS-NATO Advanced Research Workshop on General & Applied Aspects of Halophilic Microorganisms, Alicante, Spain, 1989.

Office of Naval Research Archaebacteria Meeting, Williamsburg, Virginia, 1989.

RNA Relics Meeting, University of California, San Francisco, 1989.

First Canadian Institute for Advanced Research Evolutionary Biology Meeting for Graduate Students, Montreal, Quebec, 1989.

Thirtieth Tryptophan-Plus Conference, Asilomar, California, 1990.

Fourth International Conference on Retinal Proteins, Santa Cruz, California, 1990.

Fourth International Congress of Systematic and Evolutionary Biology, College Park, Maryland, 1990.

Molecular Evolution Course, Wood's Hole, Massachusetts, 1990.

American Society for Microbiology Symposium, Dallas, Texas, 1991.

Society for General Microbiology Symposium, Edinburgh, Scotland, 1991.

Annual Symposium of the Biochemical Society of the U.K., Edinburgh, Scotland, 1991.

Canadian Institute for Advanced Research Genome Evolution Meeting, Quebec City, Quebec, 1991.
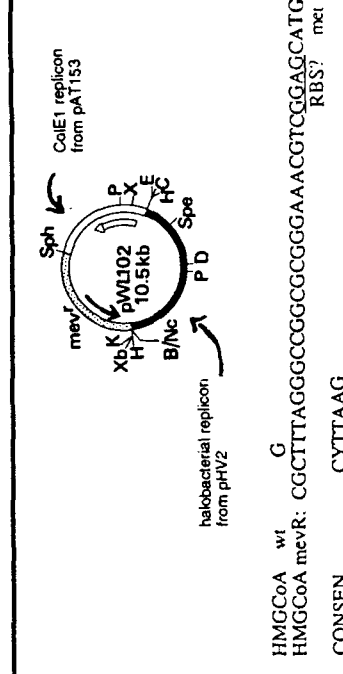
Development of Shuttle Vectors for Halobacteria

W.F. Doolittle, Dalhousie University; 1991

Objectives

- Develop transformation systems for halophilic archaebacteria

- Construct *E. coli*-halobacterial shuttle vectors

- Construct useful halobacterial host strains

- Demonstrate utility of these genetic tools

Accomplishments

- Developed efficient, simple and reliable transformation system for *Haloferax volcanii, Halobacterium halobium* and *Haloarcula hispanica* which permits uptake and expression of circular or linear, double-stranded or single stranded, self-replicating or integrating DNAs.

- Generated and characterized hundreds of *Hf. volcanii* auxotrophs, genome deletion strains and other useful mutants.

- Constructed *E. coli*-halobacterial shuttle vectors selectable by drug resistance or by complementation of amino acid auxotrophies. Demonstrated that these shuttle vectors have a broad host range within the halobacteria.

Significance

These transformation methods and shuttle vectors provide powerful tools for investigating the molecular biology of the halophilic archaebacteria by the methods of modern surrogate genetics.

HMGCoA wt   G
HMGCoA mevR: CGCTTTAGGGCCGGCGCGGGAAACGTCGGAGCATG
                                              RBS?   met
CONSEN   CYTTAAG

- Mevinolin resistance conferred by the shuttle vector pWL102 is due to an up-promotor mutation of the *Hf. volcanii* HMGCoA reductase gene.

- Constructed a set of shuttle vectors bearing UAG, UAA, and UGA suppressors derived from *Hf. volcanii* tRNAser.

- Cloned *H. halobium* bacteriorhodopsin gene cluster into *Hf. volcanii* and *Ha. hispanica* using shuttle vector.

- Mapped and characterized genes of the *Hf. volcanii arg, trp* and *his* biosynthetic pathways using shuttle vectors and genomic DNA transformations.

Principal Investigator: W. Ford Doolittle

Institute: Department of Biochemistry
                                           Faculty of Medicine
                                           Dalhousie University
                                           Halifax, Nova Scotia B3H 4H7

Grant Title: Development of Shuttle Vectors for Halobacteria

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    How many are minority
    students?   2

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